IMPORTANCE OF HIGH-RISK HUMAN PAPILLOMAVIRUS INFECTION DETECTION IN FEMALE RENAL TRANSPLANT RECIPIENTS IN THE FIRST YEAR AFTER TRANSPLANTATION

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ABSTRACT

OBJECTIVES: Most of human papilomavirus (HPV) infections are "cleared" by the immune system, however, in cases of immune system suppression infections could lead to development of malignancies. The aim of this study was to find out the frequency of HR-HPV infection in early period after renal transplantation in recipients receiving immunosuppressive therapy and to follow the progression of the infection up to one year.

METHODS: 43 female renal recipients and 79 practically healthy female individuals as a control group were enrolled in this investigation. For the detection of HPV infection patients' samples (blood and vaginal swabs) where collected two weeks after transplantation with following collection of six months and one year. Different polymerase chain reactions for HR-HPV genomic sequences detection and ELISA kit for detection of anti-HPV IgG antibodies were used.

RESULTS: In this study we show that frequency rate of HR-HPV infection has increased by the one year after transplantation from early stage of immumosuppressive therapy (from 24% to 36%). Also increase of HR-HPV load was detected over the time, showing the highest median viral load at sixth month after transplantation.

CONCLUSIONS: From the obtained data follows that it is very important to carefully monitor patients receiving immunosuppression therapy on progression of HR-HPV.

Keywords: human papillomaviruses; immunosuppression; renal recipients.

INTRODUCTION

Human papillomavirus (HPV) infections remain one of the major global burden even despite of very active use of vaccination. Prevalence rate of HPV in East Europe is about 21.4% which is much higher in comparison with the global prevalence of 11.7% [1]. Such high prevalence rate could be related with absence of HPV screening program and low vaccination uptake because of poor knowledge on HPV infection among population [2,3].

Based on association with cervical cancer and precursor lesions, HPV can be classified as high-risk (HR-HPV) and low-risk (LR-HPV) oncogenic types [4,5]. LR-HPV types, such as HPV 6 and 11, can cause common genital warts or benign hyper-proliferative lesions with very limited tendency to malignant progression, while infection with HR-HPV types, highlighting HPV 16 and 18, is associated with the occurrence of pre-malignant and malignant cervical lesions [6].

Most of HPV infections are "cleared" by the immune system and do not result in clinical diseases in healthy individuals, however, in cases of immune system suppression infections could lead to development of malignancies [7].

Considering that from one side immunosuppressive therapy has improved long-term graft and patient survival after renal transplantation, from the other side it increases the cumulative occurrence of (pre)malignancies, especially those associated with viral infections [8–10]. Declined cell-mediated immunity caused by the use of immunosuppressive therapy as the result could increase risk for HPV related anogenital (pre)malignancies in renal transplant recipients, especially in countries where the prevalence of HPV is high. Previous studies have shown dramatic increase of HR-HPV infection up to 27% [11,12].

THE OBJECTIVE of this study was to find out the frequency of HR-HPV infection in early period after renal transplantation in Latvian recipients and to follow the progression of the infection up to one year.

METHODS

43 female renal recipients (median age of 48; IQR = 39-58), who received kidney allograft during 2013-2015 and 79 practically healthy female individuals (median age of 48; IQR = 42-57), who were visiting gynaecologist for preventive examination, as a control group were enrolled in this investigation. For the early detection of HPV infection patients' samples (whole blood and vaginal swabs) where collected two weeks after transplantation with following collection of six months and one year to receive data on later periods. Chronic glomerulonephritis (7%), hypertensive nephropathy (21%), chronic interstitial nephritis (26%) and polycystic kidney disease (26%) were the most common reasons for the subsequent transplantation. All patients had received induction immunosuppression therapy with monoclonal or polyclonal antibodies and steroid bolus course. Initially immunosuppressive therapy consisted of glucocorticoids (Prednizolone tapered down to 5 mg per day during study period), anti-proliferative drugs (Cell-cept ® 2 g per day, tapered down to 1g per day if leucopenia appered) and calcineurin inhibitors (once per day tacrolimus with through level 7-10 ng/ml during first 3 months after surgery and 5-8 ng/ml thereafter or microemulsified formulation of ciclosporine with through level 150-250 ng/ml during first 3 months after surgery and therafter 100-200 ng/ml for patients transplanted in 2013).

Aliquots of 200 μl blood plasma were collected from EDTA peripheral blood samples for further serological tests. Blood plasma samples and cervical swab samples were stored at -70 ^{0}C.

DNA from cervical swab samples was extracted using phenol-chloroform method.

Beta (β)-globin PCR with appropriate primers was used to determine the quality of isolated DNA [13].

Polymerase chain reaction (PCR) with consensus primers MY9/MY11 was used for initial detection of high range HPV types (HR-HPV and LR-HPV types) [14], HPV High Risk Screen Real-TM Quant commercial qPCR kit (Sacace, Italy) - for quantitative detection of 12 types of HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59) and HPV typing with semiquantitative viral load determination was done with the Anyplex™ II HPV28 kit (Seegene, South Korea) in recipients' cervical swab DNA samples.

ELISA commercial kit (MyBioSource, USA) was used to detect IgG class antibodies against HR-HPV L1-capsids' protein in recipients' plasma (presence of these antibodies is associated with HR-HPV clearance) [15,16].

All statistical calculations and graphs were performed using GraphPad Prism software version 6.0 for Windows (GraphPad Software, San Diego, CA, USA). To test whether the collected data are normally distributed, the D'Agostino and Pearson, Anderson-Darling and ShapiroWilk normality tests were applied. Statistical differences in the prevalence of HPV infection were assessed by using Fisher's exact test. Statistical differences in viral load comparison was used Mann–Whitney U test. Because most of the data was distributed not normally, results are expressed as median and interquartile range (IQR) as dispersion characteristic, and p-value less than 0.05 ($p < 0.05$) was considered as statistically significant. Pearson and Spearman tests were used for correlation analysis.

The study was approved by the Ethics Committee of Riga Stradins University, and written consent was obtained from all patients.

RESULTS

Presence of HPV sequences detected by PCR with MY9/11 consensus primers

Recipients

Initial PCR results with consensus primers MY9/11 had shown that almost all recipients (42/43; 98%) were positive on HPV genomic sequences in cervical swab DNA samples. In 28/43 (65%) recipients' cervical swab samples viral DNA was detected two weeks after transplantation, in 25 of them viral DNA had been preserved and in 8 (total 33 recipients; 77%) additional recipients presence of HPV genomic sequences in cervical swab DNA samples appeared (total 33/43 recipients; 77%) six months after transplantation. One year after transplantation 24 recipients were still positive on HPV genomic sequences in cervical swab DNA samples (17/24 were positive after two weeks and 6 months after transplantation and another 7 recipients were positive only after time period of 6 months).

Control group

Frequency rate of HPV sequences in control group's cervical swabs DNA samples was found significantly lower (38% [30/79] vs 65% [28/43, two weeks] or 77% [33/43, six months] or 72% [31/43, one year], $p < 0.05$).

Presence of HR-HPV sequences detected by two commercial real-time PCR kits

Recipients

All HPV positive samples were tested on the presence of HR-HPV types using the Anyplex™ II HPV28 kit (Seegene, South Korea) and viral load was detected using HPV High Risk Screen Real-TM Quant commercial qPCR kit (Sacace, Italy).

Overall HR-HPV genomic sequences were found in 65% (28/43) of recipients. In early period after transplantation (two weeks) HR-HPV presence was detected in 24% (10/43) of recipients. In the following time periods, six months and one year, HR-HPV frequency increased till 26% and 36%, respectively (Figure 1). The highest HR-HPV median load was detected on the sixth months after transplantation in comparison to two weeks and one year period (3.98 log copies/10^5 cells, IQR: 1.10-6.66 versus 1.15 log copies/10^5 cells, IQR: 0.95-2.57 and 2.70 log copies/10^5 cells, IQR: 1.55-5.07 [Figure 2]).
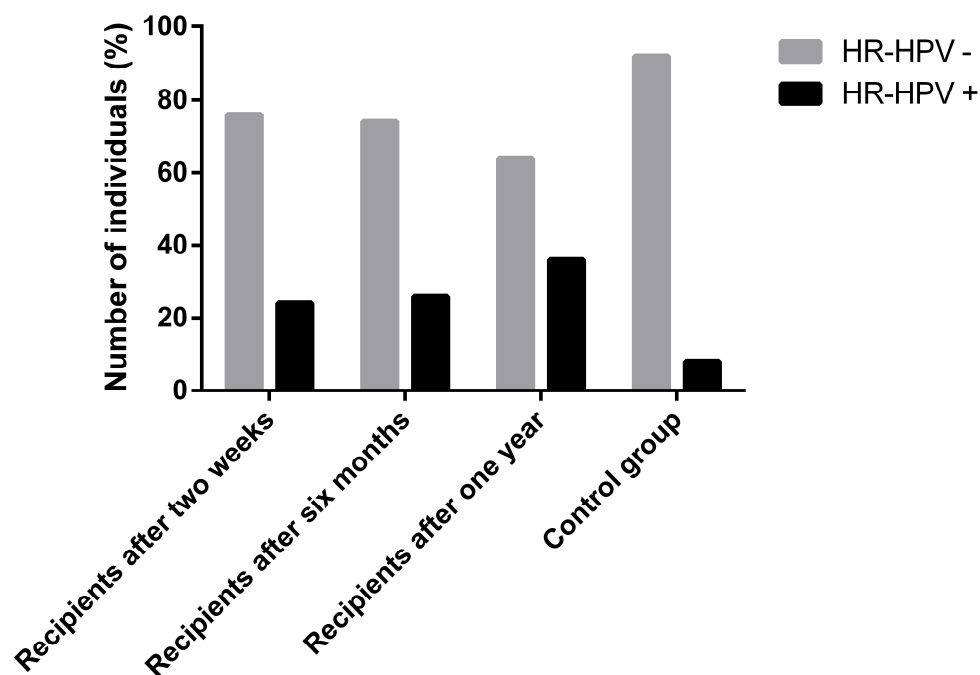
Figure 1. Presence of HR-HPV genomic sequences in recipients' group cervical swab DNA samples two weeks, six months and one year after transplantation in comparison with control group.
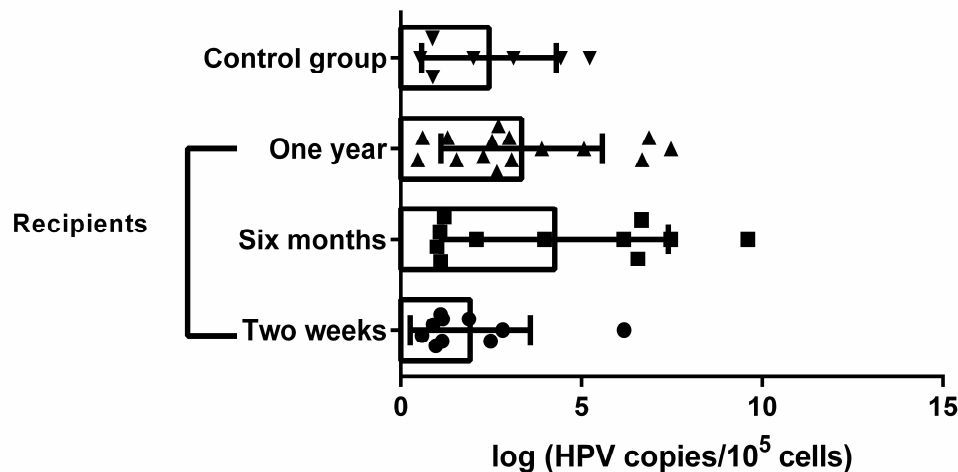
Figure 2. HR-HPV median log copies detected in recipients' group cervical swab DNA samples two weeks, six months and one year after transplantation in comparison with control group.

Among the recipients following HR-HPV types were detected: 16, 18, 31, 33, 35, 51, 56, 66, 68 and 73. The most dominant detected type was HPV-18 – in 14/42 recipients (33%) - in comparison with HPV-56 (8/42, 19%), HPV-16 (7/42, 17%), HPV-35 (7/42, 17%), HPV-51 (6/42, 14%), HPV-68 (5/42, 12%), HPV-33 (3/42, 7%), HPV-73 (2/42, 5%), HPV-66 (1/42, 2%) and HPV-31 (1/42%, 2%), however without statistical significance.

After stratification of patients' data by three age groups showed that patients in age from 20 till 40 were with higher HR-HPV loads in the period of six months after transplantation in

comparison to patients of age over 40 years (Figure 3). Statistical analysis showed that there is correlation between age and HR-HPV load in period of six months and one year after transplantation (Figure 4).
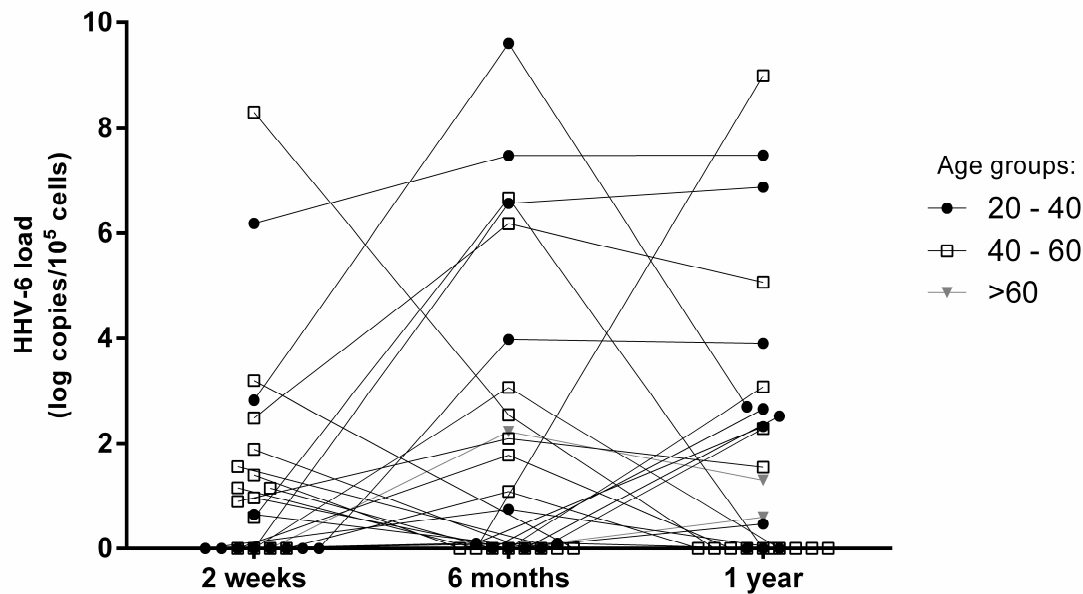
Figure 3. HR-HPV load (log copies/10^5cells) detected in each recipients' cervical swab DNA samples two weeks, six months and one year after transplantation.
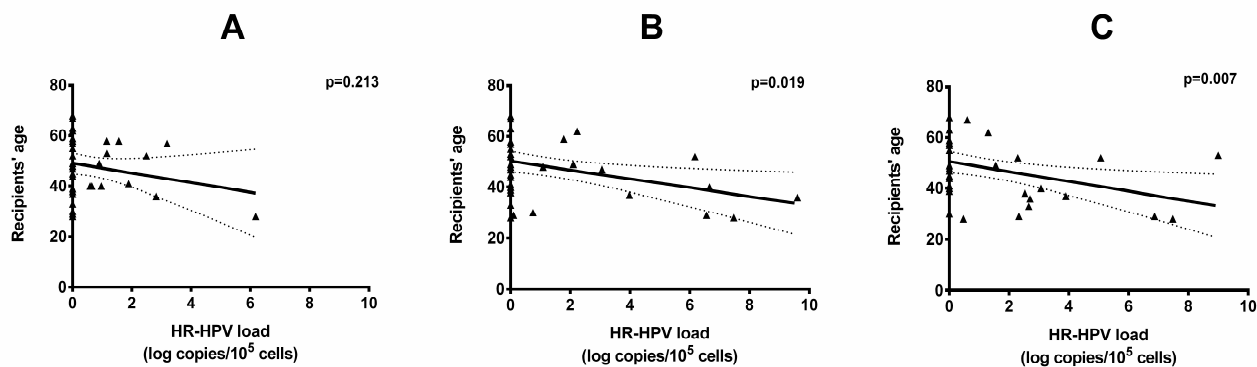
Figure 4. HR-HPV load (log copies/10^5cells) and age correlation two weeks (**A**), six months (**B**) and one year (**C**) after transplantation.

Control group

Among control group HR-HPV infection was determined in 7/79 (8%) women with the significantly lower frequency than in recipients' group (two weeks - 23%; p=0.05, six months - 26%; p=0.01 and one year - 36%; p<0.01).

Although, median HR-HPV load was higher than in recipients' group two weeks after transplantation (2.01 log copies/10^5 cells, IQR: 0.88-4.43 vs 1.15 log copies/10^5 cells, IQR: 0.95-2.57, respectively [Figure 2]).

Following HR-HPV types were detected: 16, 31, 33, 45 and 59.

Presence of IgG antibodies against HR-HPV

IgG antibodies against HR-HPV were detected in 15/43 (35%) recipients, where 10 recipients were positive during all three checking periods, two recipients only at two week period, another one was positive during first two periods and negative at one year after transplantation, as well as one recipient was positive during the last two periods and one recipient was positive only after one year post transplantation. Two recipients who became negative on HR-HPV IgG antibodies were receiving Rituximab - B lymphocytes' suppressor.

Plasma samples received from control group had shown significantly higher frequency rate of antibodies against HR-HPV (51/79, 65%, p<0.01).

Interestingly, number of the recipients positive for both, HR-HPV sequences and antibodies, was increasing over the time and number of recipients positive for antibodies only was decreased (Figure 5).
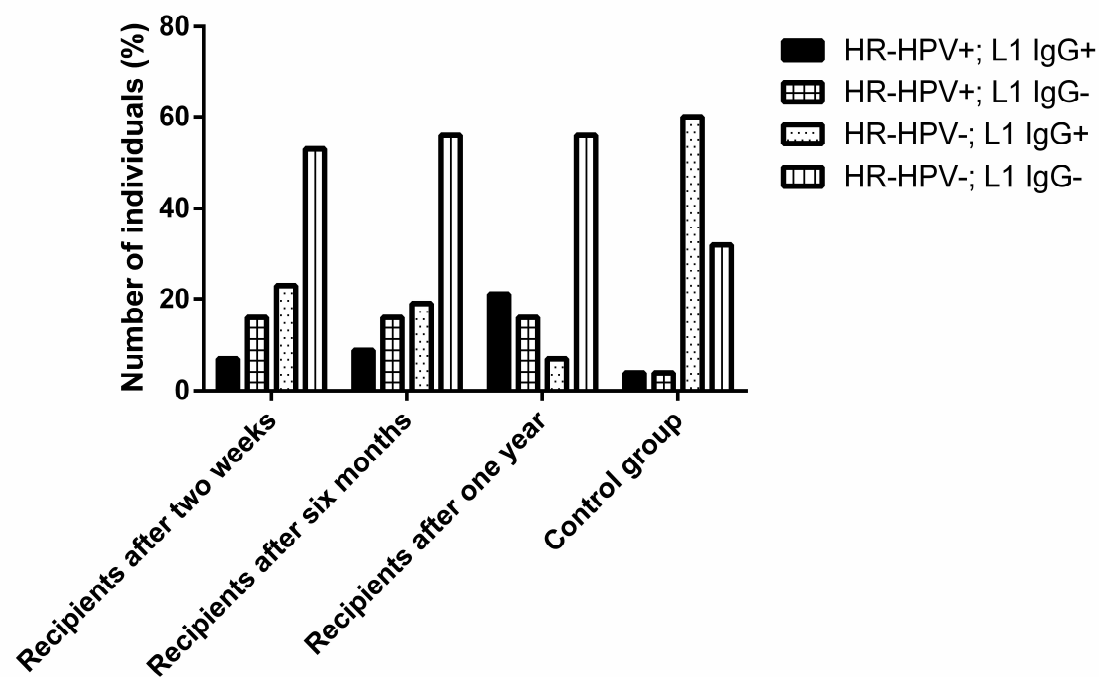
Figure 5. Comparison of HR-HPV genomic presence in cervical swab DNA samples together with presence of anti- HR-HPV L1 IgG antibodies in recipients' group of different testing time points compared with control group.

Histological results

Presence of cervical intraepithelial neoplasia (CIN) in one recipient was detected on the second week after transplantation. After six months additionally two recipients developed CIN I and after one year period in one out of three recipients with CIN I cytology results were normal. In all DNA sample obtained from cervical swabs of recipients with CIN high HR-HPV loads were detected and in two of them co-infection with several HPV types (on the second week - 1st recipient: HPV-68+HPV-18; 0.9 log copies/10^5 cells; on the sixth month - 1st recipient: HPV-73; 2.1 log copies/10^5 cells, 2nd recipient: HPV-51; 9.6 log copies/10^5 cells, 3rd recipient: HPV-16+HPV-56; 7.47 log copies/10^5 cells; after one year - 1st recipient: HPV-68+HPV-73; 1.55 log copies/10^5 cells, 2nd recipient: HPV-51+HPV-35+HPV-18; 2.7 log copies/10^5 cells [cytology results: normal], 3rd recipient: HPV-16+HPV-56; 7.48 log copies/10^5 cells) (Figure 4). Antibodies against HR-HPV among these three recipients during all three testing points were detected only in the 1st recipient's plasma samples - other two recipients were antibody negative (Figure 6).
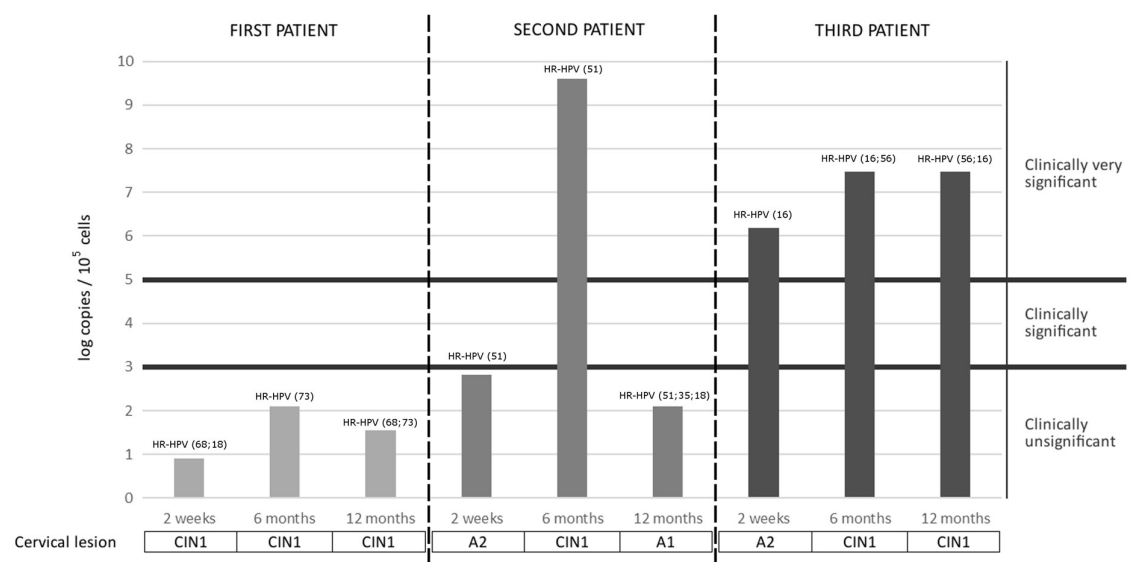
Figure 6. Progression of CIN development in three recipients over the time in comparison to HR-HPV load with qPCR result interpretation provided by manufacturers' protocol (Sacace, Italy).

Cytology results of other recipients had shown normal epithelium or such pathologies like atrophy, colpitis and cervicitis.

Among control group individuals any CIN case was detected, however, one individual with very high HPV-16 load atypical squamous cells of unknown significance (A2-ASCUS) were ascertained.

DISCUSSION

HPV persistent infection is strongly associated with development of cervical cancer [17]. Despite of development of new vaccines and increased use of them worldwide, HR-HPV infections remain one of the major global burden. This could be related with several circumstances like low uptake of vaccine in some countries and absence of HPV screening programs, as well as, that nowadays vaccines do not provide defense from all HPV types. In Latvia HPV vaccination as routine immunization approach was included in the National Health Care Program in 2010, however, uptake of the vaccine is shown to be not sufficient [3].

The HPV clearance from host's organism requires an adequate immune response, therefore, immunocompromised individuals could be in the risk group of HPV persistent infection development [18]. Because of received immunosuppressive therapy, renal allograft recipients in particular are at high risk of developing persistent infection and HPV associated cancer [19]. Another crucial factor that has an effect on the HPV infection is the recipients median age - 48,

IQR= 39-58. This means that they were not vaccinated, because, as was written above, HPV vaccination was included in Latvian National Health Care Program in 2010 and should be done in childhood till the age of 15.

Results of this research show significantly higher frequency rate of HR-HPV infection in renal transplant recipients even in early period after transplantation (two weeks - 24%; p=0.05, six months - 26%; p=0.01 and one year - 36%; p<0.01) in comparison with control group (8%). These results show the growing tendency of HR-HPV infection frequency over the time in female recipients receiving immunosuppressive therapy which indicates they are in the risk group for HPV persistent infection development and possible development of cervical cancer.

Fact that the highest level of HR-HPV load was detected in recipients' DNA samples six months after transplantation in comparison with control group (median 3.98 log copies/10^5 cells, IQR: 1.10-6.66 against 2.01 log copies/10^5 cells, IQR: 0.88-4.43) is another evidence showing immunosuppression as a high risk factor of development of HPV persistent infection. The increase of HR-HPV viral load detected in recipients' samples from two week to six months (from 1.15 log copies/10^5 cells, IQR: 0.95-2.57 to 3.98 log copies/10^5 cells, IQR: 1.10-6.66, p<0.07) could be related to deepening of immune system suppression because the mean CD4/CD8 ratio has dropped from 1.95±1.07 till 1.51±0.87. However, it is not clear why median viral load has started to decrease on the one year period after transplantation (till 2.70 log copies/10^5 cells, IQR: 1.55-5.07). It may be related to slight lowering of immunosuppressive drugs dosages.

Significant correlation between age of recipients and HR-HPV load was found only in period of six months and one year after transplantation (Figure 4), which is another evidence of immunosuppressive therapy major impact on HR-HPV infection progression as in these periods of time recipients' immunosuppression reaches maximum effect. Also, in recipients'

group of age between 20 and 40 were found higher viral loads and together with correlation results indicate that this group could be in higher risk of HR-HPV persistent infection development which plays an important role in cervical cancer development (Figure 3-4).

Higher frequency rate of IgG antibodies against HR-HPV L1 capsid protein in control group rather than in recipients' group (65% vs 35%, p<0.05) is another evidence that immunosupression plays important role in development of persistent HR-HPV infection. Also, in some recipients who had received Rituximab, which inhibit B lymphocytes' activity, absence of previously detected antibodies against HR-HPV in early period after transplantation - is found. It could indicate on crucial role of immunosuppressive therapy, including Rituximab, in suppression of humoral immune response against HPV infection.

Interesting finding is that 14% of recipients who were previously negative for the presence of HR-HPV genomic sequences but positive for the presence of antibodies against HR-HPV L1 protein became positive for viral sequences in the next observation period after transplantation (Figure 5). This could indicate on HR-HPV reactivation and supports hypothesis that HPV could has latent phase. It is still not clear how HPV creates latency, only on the mouse model had been shown that as long as it remains in basal layer's stem cells, viral replication does not occur but HPV become active only in differentiated cells [20]. In the case of HPV latent infection presence of virus-specific antibodies could not be considered as a marker of HPV clearance [21]. To evaluate development of HPV infection within time more specific markers which could indicate on the HPV latency need to be found.

Presence of cervical intraepithelial neoplasia is detected only in recipients' group and in all three cases HR-HPV infection is found. Also, high viral loads are detected in those patients (9.6, 7.48 and 2.1 log copies/10^5 cells). In these cases two facts should be pointed out: 1) in one recipient with high HPV load (9.6 log copies/10^5 cells) six months after transplantation

after one year period clearance of CIN I followed with viral load decrease to 2.7 log copies/10^5 cells is found; 2) another recipient shows presence of CIN I during all three testing points with the low viral load (0.9, 2.1 and 1.55 log copies/10^5 cells) and is also positive for antibodies against HR-HPV. First fact is showing that HR-HPV load could be useful marker in progression of HPV associated CIN development. Second fact points out on the presence of antibodies as a positive marker which indicates on the lowering of HPV load.

In this study we show that frequency rate of HR-HPV infection has increased by the one year after transplantation from early stage of immunosuppressive therapy (from 24% to 36%). Together with increase in viral load (highest median is detected on sixth months after transplantation, 3.98 log copies/10^5 cells, IQR: 1.10-6.66) these results indicate that renal transplant female recipients are at high risk of development HPV associated cervical intraepithelial neoplasia of different grade and possibly even cervical cancer. Therefore, all female recipients need to be more carefully monitored (twice a year during first two years after surgery) for HPV infection and development of CIN from the beginning of immunosuppressive treatment application. The presence of several cases of CIN I in recipients' group but not in control group could support this statement. Early detection of HR-HPV infection in female renal transplant recipients could help to prevent development of cervical cancer in the future.

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